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INVENTOR(S): Roberto Polakiewicz
Jiong Wu
Yu Li

TITLE: Antibodies Specific for Phosphorylated Insulin Receptor Substrate-1/2 (SER1101/SER1149) and Uses Thereof

ATTORNEY: JAMES GREGORY CULLEM, Reg. No. 43,569
Intellectual Property Counsel
CELL SIGNALING TECHNOLOGY, INC.
166B Cummings Center
Beverly, MA 01915
Telephone: (978) 867-2311
PAIR Customer No.: 31012

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ANTIBODIES SPECIFIC FOR PHOSPHORYLATED INSULIN RECEPTOR SUBSTRATE-1/2 (SER 1101/SER1149) AND USES THEREOF

RELATED APPLICATIONS

This application claims priority to USSN 60/422,409, filed October 30, 2002, presently pending, the disclosure of which is hereby incorporated herein.

FIELD OF THE INVENTION

The invention relates generally to antibodies, and more particularly to antibodies to signal transduction proteins and their uses.

BACKGROUND OF THE INVENTION

One of the major physiological roles of insulin is to stimulate glucose transport into insulin-sensitive cells and tissues by inducing the translocation of the major insulin responsive glucose transporter, GLUT4, from an intracellular compartment to the plasma membrane. Secretion of insulin from the beta cells in the pancreas thus tightly regulates glucose homeostasis, which is critical for normal physiologic maintenance in higher animals.

Insulin resistance, which is commonly associated with prevalent type 2 diabetes, is a state in which target cells fail to respond to normal levels of circulating insulin. *See, e.g.* Saltiel *et al., Nature 414:* 799-806 (2001). This lack of response, in turn, results in hyperinsulinemia to compensate for the resistance to insulin in the prediabetic state. Subsequently, hyperglycemia develops due to the failure of the pancreatic beta cells to produce and secrete enough insulin to compensate for the imbalance in glucose metabolism. Type 2 diabetes is the most common

form of the disease, affecting 16 million people in the United States alone. (Source: American Diabetes Association, www.diabetes.org). Roughly one-third of these people remain undiagnosed. (Source: id.)

At the molecular level, insulin resistance may result from mutations or posttranslational modifications of the insulin receptor or any of its downstream targets. The identification of simple molecular explanations for insulin resistance and type 2 diabetes has so far proven difficult. Understanding the basic mechanisms of insulin resistance at the molecular level could have a great impact on finding a cure for a chronic disease like type-2 diabetes and insulin resistance that develops in other conditions such as chronic obesity and acute trauma.

The insulin receptor (IR) is a transmembrane glycoprotein, comprising an extracellular insulin-binding domain and a transmembrane tyrosine protein kinase domain that undergoes autophosphorylation following insulin binding. Auto-phosphorylation in turn activates the IR intrinsic tyrosine kinase activity and triggers phosphorylation of numerous downstream targets that ultimately mediate insulin's several biological effects.

The major targets of insulin receptor kinase are the insulin-receptor substrate (IRS) proteins IRS-1, IRS-2, IRS-3, IRS-4, the adaptor proteins Shc, Gab1, APS, p60Dok, SIRPS and c-Cbl. *See, e.g.* White, M.F., *Mol. Cell. Biochem. 182:* 3-11 (1998); Kahn et al., U.S. Patent No. 5,621,075, Issued April 15, 1997; Holgado-Madruga, *et al.*, *Nature 379:* 560-564 (1996); Sasaoka *et al.*, *J. Biol. Chem. 269:* 13689-694 (1994); Moodie *et al.*, *J. Biol. Chem. 274:* 11186-193 (1999). The C-terminal region of IRS proteins contains multiple tyrosine phosphorylation motifs that serve as docking sites for many SH2 domain-containing proteins, such as the p85 regulatory subunit of PI3K, which mediate many of the down-stream biological actions of insulin. *See, e.g.* White (1998), *supra.*

The relative roles of the different IRS proteins in insulin signaling and diabetes have been intensively studied. Gene targeting experiments have revealed that IRS proteins are essential for normal development and metabolism. *See, e.g.* Saltiel *et al.*, *Trends Cell Biol. 12:* 65-71 (2002). For example, mice lacking IRS-1 grow poorly *in utero* and remain small throughout life, but diabetes does not develop because insulin secretion increases to compensate for a mild insulin resistance. In contrast, IRS-2 null mice develop insulin resistance and beta cell failure, and die from type 2-like diabetes. These results not only suggest a critical role of IRS proteins in mediating insulin action, but also indicate that understanding the regulation of IRS proteins can provide important clues as to the causes of insulin resistance.

Protein phosphorylation is an important mechanism by which the activity of the insulin-signaling pathway, as with most signaling pathways, is regulated. A major negative regulatory mechanism for insulin action has been attributed to agents that enhance serine or threonine (Ser/Thr) phosphorylation of either the IR itself, or of its downstream effectors. Ser/Thr phosphorylation reduces the tyrosine kinase activity of the IR, and thus its ability to phosphorylate substrate proteins. For example, insulin's counter regulators, such as epinephrine and glucagons, increase cAMP levels in the cell, thereby activating the cAMP-dependent protein kinase (PKA) and increasing the Ser/Thr phosphorylation of the insulin receptor, which results in an insulin-resistant state. Similarly, the general inhibitor of protein phosphatases, okadaic acid, inhibits tyrosine phosphorylation of IRS-1, while increasing its phosphorylated Ser/Thr content. Tumor necrosis factor-α, a known mediator of insulin resistance during infection, tumor cachexia, and obesity all cause similar effects.

It has been postulated that phosphorylation of serine residues significantly reduces the ability of IRS-1 and IRS-2 to interact with, and become, tyrosine phosphorylated by the IR. *See, e.g.* White (1998),

supra.; Zick Y., *Trends Cell Biol. 11:* 437-441 (2001); Saltiel *et al.* (2002), *supra.* Phosphorylation of IRS at Ser/Thr residues and the consequent inactivation of insulin signaling can be triggered by prolonged exposure to insulin itself or by cross-desensitization with other factors that provoke IRS phosphorylation, *e.g.* PDGF, IGF-1 endothelin, or TNFα.

However, there is mounting evidence indicating that each stimulus can desensitize IRS-1 and IRS-2 function through a different mechanism, implying the phosphorylation of different sites in the IRS proteins. Indeed, IRS proteins contain over 30 potential Ser/Thr phosphorylation sites for kinases like PKA, PKC, mitogen-activated protein kinase (MAPK), Akt (PKB) and others. *See, e.g.* White (1998), *supra.*; Zick, *supra.*; Saltiel *et al.* (2002), *supra.* For example, stimulators of PKC, such as phorbol esters or endothelin, induce the activity of MAPK, which then phosphorylates IRS-1 at Ser612. *See, e.g.* Jiang *et al.*, *Diabetes 48:* 1120-1130 (1999); Mothe *et al.*, *J. Biol. Chem. 271:* 9351-9356 (1996). This phosphorylation event reduces IRS-1 tyrosine phosphorylation by the IR, as well as IRS-1 association with PI3K. In contrast, the inhibitory effects of PDGF were reported to not require Ser612, but instead phosphorylation of three other serine residues (632, 662, and 731) were involved through a mechanism implicating PI3K/Akt and mTOR pathway. *See* Li J., *J. Biol. Chem. 274:* 9351-9356 (1999). Phospho-specific antibodies to certain of these IRS-1 phosphorylation sites are commercially available. (*See, e.g.* Upstate Biotechnology, Inc., Cat. No. 07-247(Ser307); Cell Signaling Technology, Inc., Cat. Nos. 2388, 2386 (Ser 636/639 and 612); BioSource, Inc., Cat. No. 44-550 (Ser616)).

The protein kinase Akt, however, has been implicated in positively modulating IRS function by preventing its rapid tyrosine phosphorylation. *See* Zick, *supra.* The action of Akt appears to involve four possible serine residues in human IRS (270, 307, 330, and 383), but the evidence on which of these sites is essential for negative or positive regulation by Akt

is inconclusive. Other recent studies suggest that Ser312 (307 in the mouse) in IRS-1 is actually regulated by TNF-α through activation and direct phosphorylation by the Jun-terminal kinase (JNK), a kinase of the MAPK family. *See, e.g.* Aguirre *et al.*, *J. Biol. Chem. 275:* 9047-9054 (2000); Rui *et al.*, *J. Clin. Invest. 107:* 181-189 (2001); Aguirre *et al.*, *J. Biol. Chem. 277:* 1531-1537 (2002). This finding could explain the well-documented insulin resistance that is provoked by acute stress and mediated through TNF-α action.

Taken together, these results underscore the important role that IRS-1 Ser/Thr phosphorylation plays in type 2 diabetes. However, the precise mechanisms by which particular signaling events are mediated by IRS-1 phosphorylation remain unclear, and the serine or threonine residues relevant to such mechanisms remain unidentified. For example, chronic and acute elevation of plasma free fatty acid is commonly linked to impaired insulin-mediated glucose uptake. See Griffin *et al.*, *Diabetes 48:* 1270-1274 (1999). The mechanisms underlying these changes in glucose transport are unknown, but may include changes in insulin signaling. It has been shown that protein kinase C (PKC) theta protein levels, one of the major PKC isoforms expressed in skeletal muscle, are elevated in insulin resistant humans and rats. Moreover, PKC theta activity is enhanced by elevated plasma free-fatty acids. *See* Itani *et al.*, *Metabolism 50:* 553-557 (2001); Qu *et al.*, *J. Endocrinol. 162:* 207-214 (1999); Chalfant *et al.*, *Endocrinology 141:* 2773-2778 (2000). PKC theta thus represents a potential therapeutic target for modulating insulin signaling in obesity-driven insulin resistance, but the mechanism of its action remains unclear.

Accordingly, there remains a need for the identification of Ser/Thr phosphorylation sites in IRS-1 and IRS-2 that are essential for the inhibition of insulin signaling leading to insulin resistance and type 2 diabetes. The production of phospho-specific antibodies directed at such

sites would greatly facilitate the elucidation of critical phosphorylated Ser/Thr residues in IRS-1 and IRS-2, particularly in the context of the diverse pathological circumstances and pathways that cause insulin resistance. Such antibodies would be valuable tools for the early diagnosis of type-2 diabetes and other conditions involving insulin resistance, as well as for drug discovery programs aimed at identifying new compounds for the restoration of insulin-sensitivity in diabetic individuals.

SUMMARY OF THE INVENTION

The invention discloses a novel human IRS-1 phosphorylation site, serine 1101 (Ser1101), and a homologous novel phosphorylation site, serine 1149 (Ser1149) in human IRS-2, as well as homologous sites in mouse IRS-1 (Ser1095) and IRS-2 (Ser1138), and provides antibodies that selectively bind to IRS-1 and/or IRS-2 when phosphorylated at these novel sites. Also provided are methods for determining the phosphorylation of IRS-1 and/or IRS-2 in a biological sample, profiling IRS-1 and/or IRS-2 activation in a test tissue, and identifying a compound that modulates phosphorylation of IRS-1 and/or IRS-2, by using a detectable reagent, such as the disclosed antibodies, that binds to IRS-1 and/or IRS-2 when phosphorylated at Ser1101 and/or Ser1149, respectively. In preferred embodiments, the sample or test tissue is taken from a subject potentially having or suspected of having type 2 diabetes.

The invention further discloses that the novel human IRS-1 serine 1101 site identified herein (corresponding to ser1149 in IRS-2) is phosphorylated by protein kinase C theta (PKC theta (θ)). Accordingly, the invention also provides methods for determining the activity of PKC theta in a biological sample, profiling PKC theta activation in a test tissue, and identifying a compound that modulates PKC theta activity, by using a detectable reagent, such as the disclosed antibodies, that binds to IRS-1

when phosphorylated at Ser1101 and/or to IRS-2 when phosphorylated at Ser1149. In preferred embodiments, the sample or test tissue is taken from a subject potentially having, or suspected of having, a disease characterized by, or associated with, altered PKC theta activity, such as type 2 diabetes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 – is the amino acid sequence (1-letter code) of human IRS-1 (SEQ ID NO: 1) (SwissProt Acc# P35568). Ser1101 is underlined, and the peptide sequence encompassing Ser1101 and corresponding to the immunogen used to generate an exemplary IRS-1 (Ser1101)/IRS-2 (Ser1149) phosphospecific antibody is indicated in bold (*see* Example 1).

Fig. 2 – is the amino acid sequence (1-letter code) of human IRS-2 (SEQ ID NO: 2) (SwissProt Acc# Q9Y4H2). Ser1149 is underlined, and the peptide sequence encompassing Ser1149 and indicated in bold corresponds to the nearly-identical sequence in human IRS-1 (as shown in Figure 1) that was used as an immunogen in Example 1.

Figs. 3-5 – are Western blot analyses using phospho-IRS-1 (Ser1101, Ser330, or Ser307) polyclonal antibodies and CHO cells overexpressing the IR and IRS-1 (Figs. 2, 5 and 6), 3T3L1 adipocytes (Fig. 3) and differentiated L6 myocytes (Fig. 4). Cells were stimulated with insulin (200 nM), and TNFα (100 nM), as indicated, for 5 minutes (Figs. 4,5), or for the times indicated (Figs 2-3). Cell extracts were prepared and subjected directly to SDS-PAGE and immunoblotting with the antibodies as indicated (Figs. 2, 3, 6), or alternatively, IRS-1 was immunoprecipitated with an IRS-1 specific antibody and immunoblotted (Figs. 3-5). Extracts from the same treated cells were immunoblotted with antibodies against phospho-Akt (Ser473); phospho-MAPK (Thr202/Tyr204); phosphotyrosine (PY-100); and total IRS-1, which

served as controls for verifying the stimulus and protein loading of the gels.

Fig. 6 – is a Western blot analysis of IRS-1 protein carrying an HA epitope tagged after immunoprecipitation with an anti-HA antibody, and probed with the phospho-IRS-1/2 (Ser1101/Ser1149) antibody of the invention, a phosphotyrosine (pY-100) antibody), and anti-HA antibody, which served as controls for verifying the insulin stimulus (induction of IRS-1 tyrosine phoshorylation) and equivalent amounts of HA-tagged IRS-1 protein. Cells were previously transfected with HA-tagged IRS-1 wild type and IRS-1 carrying a mutation of Ser1101 to Alanine.

Fig. 7 – is a Western blot analysis of IRS-1 protein immunoprecipitated with a total anti-IRS-1 antibody from liver extracts of lean and obese/diabetic Zucker rats and probed with the phospho-IRS-1 (Ser1101)/IRS-2 (Ser1149) antibody of the invention.

Fig. 8 – shows a comparison of amino acid sequences around Ser1101 and Ser1149 in human IRS-1 and IRS-2, respectively; below the sequences is a Western blot analysis of IRS-2 protein extracted from CHO cells overexpressing the IRS-2 protein. Phosphorylation of IRS-2 at Ser1149 was induced by insulin treatment at different times as indicated. Total Akt protein immunoblots served as loading controls.

Figs. 9 & 10 – are Western blot analyses using phospho-IRS-1 (Ser1101)/IRS-2(Ser1149) polyclonal antibodies of the invention and CHO cells overexpressing the IR and IRS-1. Cells were stimulated with insulin (200 nM), TPA (200 nM), PDGF (100nM) and TNFα (100 nM), as indicated, for 5 minutes. Cell extracts were prepared and subjected directly to SDS-PAGE and immunoblotting with the antibodies as indicated. Extracts from the same treated cells were immunoblotted with antibodies against phospho-Akt (Ser473); phospho-MAPK (Thr202/Tyr204); phosphotyrosine (PY-100); phospho-PKC(pan) and total

IRS-1, which served as controls for verifying the stimulus and protein loading of the gels. In Fig. 10, cells were exposed to the following inhibitors prior to insulin stimulus: wortmannin (wt); PD98059 (PD); genistein (gen); PP2; SU6686 (SU); bis-indoleimide (bis); H89; KN62.

Fig. 11 – is a Western Blot analysis indicating the *in vitro* phosphorylation of IRS-1 at Ser1101 by PKC theta. Active, purified PKC isoforms as indicated, including PKC theta and Akt kinases were used to phosphorylate, *in vitro*, a GST-IRS-1 C-terminus fusion protein containing Ser1101, full length IRS-1 immunoprecipitated from unstimulated CHO-IR/IRS-1 cell extracts, or a GSK3 fusion protein (known substrate for Akt) as a control of Akt activity. In panel b, PKC theta and Akt were used to phosphorylated *in vitro*, full length IRS-1 immunoprecipitated from CHO cell over expressing IRS-1 with an IRS-1 antibody.

Fig. 12 – is a Western blot analysis describing the effects *in vivo* of a catalytically active PKC theta expression (panel a), or dominant negative PKC theta (panel b) on the phoshorylation of IRS-1 at Ser1101. NIH3T3 cells were co-transfected with expression constructs for these two PKC theta mutant proteins together with wild-type HA-tagged IRS-1, and stimulated with 200 nM insulin as indicated. IRS-1 proteins were immunoprecipitated with an anti-HA antibody and immunoblotted with phospho-IRS-1 (Ser1101)/IRS-2(Ser1149) antibody of the invention, with a phosphotyrosine antibody (pY-100) to control for the efficacy of the insulin treatment, or with the anti-HA antibody to control for the amount of protein in the assay.

Fig. 13 – is the amino acid sequence (1-letter code) of mouse IRS-1 (SEQ ID NO: 3) (SwissProt Acc# P35569). Ser1095 is underlined, and the peptide sequence encompassing Ser1095 and corresponding to the highly-homologous sequence of the human IRS-1 (Ser1101) phosphorylation site is indicated in bold (*see* Fig. 1).

Fig. 14 – is the amino acid sequence (1-letter code) of mouse IRS-2 (SEQ ID NO: 4) (SwissProt Acc# P81122). Ser1138 is underlined, and the peptide sequence encompassing Ser1138 and indicated in bold corresponds to the highly-homologous sequence of the murine IRS-1 (Ser1095) and human IRS-2 (Ser1149) phosphorylation sites (*see* Figs, 13 and 2).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, a novel site of IRS-1 phosphorylation, serine 1101 (Ser1101) in the human sequence (*see* Figure 1), has now been identified. A highly homologous novel site of IRS-2 phosphorylation, serine 1149 (Ser1149) in the human sequence (*see* Figure 2), has also now been identified, as well as novel, highly-homologous sites in mouse IRS-1 and IRS-2. Although IRS-1 phosphorylation at various serine residues has previously been described (Zick, *supra.* (serines 270, 307, 330, 383); Aguirre *et al.* (2000), *supra.* (serine 312); Li, *supra.* (serines 612, 632, 662, 731)), the serine phosphorylation site (Ser1101) disclosed herein has previously been unknown. Equivalent phosphorylation sites in IRS-2 have not been described.

The newly identified Ser1101 phosphorylation site was identified/predicted by analyzing the human IRS-1 amino acid sequence with the ScanSite program (http://scansite.mit.edu) (*see also* Yaffe *et al.*, *Nat Biotechnol. 19(4):* 348-53 (2001)). This algorithm searches for motifs within proteins that are likely to be phosphorylated by specific protein kinases or bind to domains such as SH2 domains, 14-3-3 domains or PDZ domains. Optimal phosphorylation sites for particular serine/threonine protein kinases or tyrosine protein kinases are predicted using a matrix of selectivity values for amino acids at each position

relative to the phosphorylation site, as determined from the oriented peptide library technique described by Songyang *et al.*, Current Biology 4: 973-982 (1994) and Songyang *et al.*, Nature 373: 536-539 (1995).

Analysis of the human IRS-1 protein sequence with ScanSite revealed several potential Ser/Thr phosphorylation sites, including putative Akt, Casein Kinase, GSK3 and PKCz sites. Attention was focused on the Akt sites due to the importance of the PI3K/Akt cascade in insulin/IRS signaling. The three sites identified, numbered according to human IRS-1 (Figure 1, SEQ ID NO: 1), all include a typical Akt consensus (RRXRXXS) motif sequence, and are (phosphorylated serine indicated by bold *S):

Ser307: TRRSRTE***S**ITATSPA (SEQ ID NO: 5)
Ser330: SFRVRAS***S**DGEGTMS (SEQ ID NO: 6)
Ser1101: GCRRRHS***S**ETFSSTP (SEQ ID NO: 7).

The potential role of Ser307 and Ser330 in IRS-1 phosphorylation has been reported. *See* Paz *et al.*, *J. Biol. Chem. 274:* 28816- 28822 (1999). However, the role of Ser1101 has not. Sequence comparison of the IRS-1 (Ser1101) phosphorylation site with human IRS-2 (SEQ ID NO: 2) identified a novel and highly homologous phosphorylation site at Ser1149 in IRS-2:

Ser1149: GGRRRHS***S**ETFSSTT (SEQ ID NO: 8).

Further sequence comparison of the human IRS-1 (Ser1101) and IRS-2 (Ser1149) sites, respectively, with the mouse IRS-1 and IRS-2 sequences identified novel and highly homologous phosphorylation sites at Ser1095 (IRS-1) and Ser1138 (IRS-2) in the mouse proteins as follows:

Ser1095: GCRRRHS***S**ETFSAPT (SEQ ID NO: 9);
Ser1138: GGRRRHS***S**ETFSSTT (SEQ ID NO: 10).

Phosphorylation of human IRS-1 at Ser1101 and IRS-2 at Ser1149 was confirmed using exemplary phospho-specific antibodies of the invention (*see* Examples). As a result of this discovery, peptide antigens may now be designed to raise phospho-specific antibodies that bind IRS-1 only when phosphorylated at Ser1101 and/or IRS-2 only when phosphorylated at Ser1149 in the human peptides sequences, and/or to the equivalent and highly-homologous sites in mouse IRS-1 and IRS-2 (Ser1095, Ser1138), or other species, such as rat (Ser1100 in IRS-1).

In further accordance with the present invention, is has now been shown that the novel IRS-1 (Ser1101) phosphorylation site is, in fact, phosphorylated by protein kinase C theta (PKC theta), the activity of which is increased in diabetic patients and insulin resistant animals (*see* Itani *et al.*, *supra.*; Chalfant *et al.*, *supra.*; Qu *et al.*, *supra.*) Phosphorylation of IRS-1 at serine residues has been shown to correlate mostly with desensitization of the insulin stimulus, and, therefore, phosphorylation of IRS-1 at Ser1101 may represent an important surrogate marker of insulin resistance, which develops as a consequence of Type 2 diabetes. Given the high homology between the IRS-1 (Ser1101) and IRS-2 (Ser1149) phosphorylation sites (the latter is identical to the former except for two residues) it is expected that PKC theta will also phosphorylate IRS-2 (Ser1149). Thus, phosphorylation of IRS-2 (Ser1149) may also represent an important surrogate marker of insulin resistance in Type 2 diabetes.

Accordingly, the invention provides, in part, phospho-specific antibodies that bind to human IRS-1 and/or IRS-2 only when phosphorylated at serine 1101 and serine 1149, respectively, and do not recognize the unphosphorylated forms, or other IRS-1 or IRS-2 phosphorylation sites. Also provided are methods of using a detectable reagent that binds to phosphorylated IRS-1/2 (Ser1101/Ser1149) to detect IRS-1/2 phosphorylation and activation in a biological sample or

test tissue potentially containing, or suspected of containing, phosphorylated IRS-1 and/or IRS-2, or having altered insulin signaling or IRS-1/2 activity, as further described below. The invention also provides, in part, methods of using a detectable reagent that binds to phosphorylated IRS-1/2 (Ser1101/Ser1149) to detect PKC theta activity in a biological sample, test tissue, or subject potentially having, or suspected of having, altered insulin signaling, PKC theta activity, or IRS-1 phosphorylation, as further described below. In preferred embodiments, the detectable reagent is at least one IRS-1/2 (Ser1101/Ser1149) antibody of the invention, and the sample or tissue is taken from a subject potentially having, or suspected of having, type 2 diabetes.

The further aspects, advantages, and embodiments of the invention are described in more detail below. All references cited herein are hereby incorporated by reference.

A. Antibodies and Cell Lines

IRS-1/2 phospho-specific antibodies of the invention bind to human IRS-1 only when phosphorylated at Ser1101 and/or to human IRS-2 only when phosphorylated at Ser1149, and do not substantially bind to IRS-1 or IRS-2 when not phosphorylated at these respective residues, nor to IRS-1 or IRS-2 when phosphorylated at other serine residues. The IRS-1/IRS-2 antibodies also bind highly homologous and equivalent IRS-1 and/or IRS-2 sites in other species, for example mouse IRS-1 (Ser1095) and/or IRS-2 (Ser1138), respectively, as disclosed herein. The IRS-1/2 antibodies of the invention include (a) monoclonal antibodies that bind phospho-IRS-1 (Ser1101) and/or phospho-IRS-2 (Ser1149), (b) polyclonal antibodies which bind to phospho-IRS-1 (Ser1101) and/or phospho-IRS-2 (Ser1149), and (c) antibodies (monoclonal or polyclonal) which specifically bind to the phospho-antigen (or more preferably the epitope) bound by the exemplary IRS-1/2(Ser1101/Ser1149) antibodies

disclosed in the Examples herein, (d) antibodies as described in (a)-(c) above that bind equivalent phosphorylation IRS-1 and/or IRS-2 sites in other species (e.g. mouse, rat), as disclosed herein, and (e) fragments of (a), (b), (c), or (d) above which bind to the antigen (or more preferably the epitope) bound by the exemplary antibodies disclosed herein. Such antibodies and antibody fragments may be produced by a variety of techniques well known in the art, as discussed below. Antibodies that bind to the phosphorylated epitope (*i.e.*, the specific binding site) bound by the exemplary IRS-1/2 (Ser1101/Ser1149) antibodies of the Examples herein can be identified in accordance with known techniques, such as their ability to compete with labeled IRS-1/2 antibodies in a competitive binding assay.

The preferred epitopic site of the human IRS-1/2 (Ser1101/Ser1149) antibodies of the invention is a peptide fragment consisting essentially of about 11 to 17 amino acids including the phosphorylated serine 1101 (in the case of IRS-1) or serine 1149 (in the case of IRS-2), wherein about 5 to 8 amino acids are positioned on each side of the serine phosphorylation site (for example, residues 1095-1108 of SEQ ID NO: 1, or residues 1143-1156 of SEQ ID NO: 2). This epitopic site, for example, corresponds to the following equivalent murine sites: residues 1089-1102 of SEQ ID NO: 3 (mouse IRS-1) (encompassing Ser1095) and residues 1132-1145 of SEQ ID NO: 4 (mouse IRS-2) (encompassing Ser1138).

The invention is not limited to IRS-1/2 antibodies, but includes equivalent molecules, such as protein binding domains or nucleic acid aptamers, which bind, in a phospho-specific manner, to essentially the same phosphorylated epitope to which the IRS-1/2 antibodies of the invention bind. *See, e.g.*, Neuberger *et al., Nature 312:* 604 (1984). Such equivalent non-antibody reagents may be suitably employed in the methods of the invention further described below.

The term "antibody" or "antibodies" as used herein refers to all types of immunoglobulins, including IgG, IgM, IgA, IgD, and IgE. The antibodies may be monoclonal or polyclonal and may be of any species of origin, including (for example) mouse, rat, rabbit, horse, or human, or may be chimeric antibodies. *See, e.g.*, M. Walker *et al.*, *Molec. Immunol. 26:* 403-11 (1989); Morrision *et al.*, *Proc. Nat'l. Acad. Sci. 81:* 6851 (1984); Neuberger *et al.*, *Nature 312:* 604 (1984)). The antibodies may be recombinant monoclonal antibodies produced according to the methods disclosed in U.S. Pat. No. 4,474,893 (Reading) or U.S. Pat. No. 4,816,567 (Cabilly *et al.*) The antibodies may also be chemically constructed specific antibodies made according to the method disclosed in U.S. Pat. No. 4,676,980 (Segel *et al.*)

The term "IRS-1/2 antibodies" is used interchangeably with the term "IRS-1/2 (Ser1101/Ser1149) antibodies" which means antibodies that specifically bind phospho-IRS-1 (Ser1101) and/or phospho-IRS-2 (Ser1149) (in the human sequence), both monoclonal and polyclonal, as disclosed herein. The term includes antibodies that bind equivalent and highly-homologous sites in IRS-1 and IRS-2 from other species, for example, murine IRS-1 (Ser1095) and/or IRS-2 (Ser1138). The term "does not bind" with respect to disclosed antibodies means does not substantially react with as compared to binding to phospho-IRS-1 and/or phospho-IRS-2. The term includes antibodies that bind whole protein comprising the target phosphorylation site, as well as shorter IRS-1 and/or IRS-2 polypeptides or fragments comprising the phosphorylated serine residue (e.g. a polypeptide of 5-25 or 25-50 or more residues comprising the target phosphorylation site).

The term "detectable reagent" means a molecule, including an antibody, peptide fragment, binding protein domain, etc., the binding of which to a desired target is detectable or traceable. Suitable means of detection are described below.

Polyclonal antibodies of the invention may be produced according to standard techniques by immunizing a suitable animal (*e.g.*, rabbit, goat, etc.) with an antigen encompassing phospho-Ser1101 (equivalent to phospho-Ser1149), collecting immune serum from the animal, and separating the polyclonal antibodies from the immune serum, in accordance with known procedures. In a preferred embodiment, the antigen is a phospho-peptide antigen comprising the IRS-1/2 sequence surrounding and including phospho-Ser1101/1149, respectively, the antigen being selected and constructed in accordance with well-known techniques. *See, e.g.*, ANTIBODIES: A LABORATORY MANUAL, Chapter 5, p. 75-76, Harlow & Lane Eds., Cold Spring Harbor Laboratory (1988); Czernik, *Methods In Enzymology, 201:* 264-283 (1991); Merrifield, *J. Am. Chem. Soc. 85:* 21-49 (1962)).

A particularly preferred peptide antigen, CRRRHS***S**ETFSST (SEQ ID NO: 11) (where ***S** = phosphoserine) is described in Example 1, below. It will be appreciated by those of skill in the art that longer or shorter phosphopeptide antigens may be employed. *See Id.* Polyclonal IRS-1/2 antibodies produced as described herein may be screened as further described below. This preferred antigen corresponds to the equivalent phosphorylation sites in murine IRS-1 and IRS-2 (*see* Figs. 13 and 14, respectively).

Monoclonal antibodies of the invention may be produced in a hybridoma cell line according to the well-known technique of Kohler and Milstein. *Nature 265:* 495-97 (1975); Kohler and Milstein, *Eur. J. Immunol. 6:* 511 (1976); *see also*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel *et al.* Eds. (1989). Monoclonal antibodies so produced are highly specific, and improve the selectivity and specificity of diagnostic assay methods provided by the invention. For example, a solution containing the appropriate antigen may be injected into a mouse and, after a sufficient time (in keeping with conventional techniques), the

mouse sacrificed and spleen cells obtained. The spleen cells are then immortalized by fusing them with myeloma cells, typically in the presence of polyethylene glycol, to produce hybridoma cells. The hybridoma cells are then grown in a suitable selection media, such as hypoxanthine-aminopterin-thymidine (HAT), and the supernatant screened for monoclonal antibodies having the desired specificity, as described below. The secreted antibody may be recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange or affinity chromatography, or the like.

Monoclonal Fab fragments may also be produced in *Escherichia coli* by recombinant techniques known to those skilled in the art. *See, e.g.,* W. Huse, *Science 246:* 1275-81 (1989); Mullinax *et al.*, *Proc. Nat'l Acad. Sci. 87:* 8095 (1990). If monoclonal antibodies of one isotype are preferred for a particular application, particular isotypes can be prepared directly, by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class-switch variants (Steplewski, *et al.*, *Proc. Nat'l. Acad. Sci., 82:* 8653 (1985); Spira *et al.*, *J. Immunol. Methods, 74:* 307 (1984)).

The invention also provides hybridoma clones, constructed as described above, that produce IRS-1/2 monoclonal antibodies of the invention. Similarly, the invention includes recombinant cells producing a phospho-IRS-1/2 (Ser1101/Ser1149) antibody as disclosed herein, which cells may be constructed by well known techniques; for example the antigen combining site of the monoclonal antibody can be cloned by PCR and single-chain antibodies produced as phage-displayed recombinant antibodies or soluble antibodies in *E. coli* (*see, e.g.,* ANTIBODY ENGINEERING PROTOCOLS, 1995, Humana Press, Sudhir Paul editor.)

IRS-1/2 antibodies of the invention, whether polyclonal or monoclonal, may be screened for epitope and phospho-specificity

according to standard techniques. *See, e.g. Czernik et al., Methods in Enzymology, 201:* 264-283 (1991). For example, the antibodies may be screened against the phospho and non-phospho peptide library by ELISA to ensure specificity for both the desired antigen (*i.e.* that epitope including Ser1101/Ser1149) and for reactivity only with the phosphorylated form of the antigen. Peptide competition assays may be carried out to confirm lack of reactivity with other IRS-1 and/or IRS-2 phosphoepitopes. The antibodies may also be tested by Western blotting against cell preparations containing IRS-1 and/or IRS-2, *e.g.* cell lines over-expressing IRS-1 and/or IRS-2, to confirm reactivity with the desired phosphorylated target. Specificity against the desired phosphorylated epitopes may also be examined by construction IRS-1/2 mutants lacking phosphorylatable residues at positions outside the desired epitope known to be phosphorylated, or by mutating the desired phospho-epitope and confirming lack of reactivity. IRS-1/2 antibodies of the invention may exhibit some limited cross-reactivity with non-IRS-1/2 epitopes. This is not unexpected as most antibodies exhibit some degree of cross-reactivity, and anti-peptide antibodies will often cross-react with epitopes having high homology to the immunizing peptide. *See, e.g., Czernik, supra.* Cross-reactivity with non-IRS-1/2 proteins is readily characterized by Western blotting alongside markers of known molecular weight. Amino acid sequences of cross-reacting proteins may be examined to identify sites highly homologous to the IRS-1/2 sequence surrounding Ser1101/1149.

IRS-1/2 antibodies may be further characterized via immunohistochemical (IHC) staining using normal and diseased tissues to examine IRS-1/2 phosphorylation and activation status in diseased tissue. IHC may be carried out according to well-known techniques. *See, e.g.*, ANTIBODIES: A LABORATORY MANUAL, Chapter 10, Harlow & Lane Eds., Cold Spring Harbor Laboratory (1988). Briefly, paraffin-embedded tissue

(*e.g.* tumor tissue) is prepared for immunohistochemical staining by deparaffinizing tissue sections with xylene followed by ethanol; hydrating in water then PBS; unmasking antigen by heating slide in sodium citrate buffer; incubating sections in hydrogen peroxide; blocking in blocking solution; incubating slide in primary antibody and secondary antibody; and finally detecting using ABC avidin/biotin method according to manufacturer's instructions.

IRS-1 antibodies of the invention bind to human IRS-1 and/or IRS-2 when phosphorylated at the Ser1101 and/or Ser1149 site, respectively, but are not limited only to the human species, per se. Phospho-specific antibodies that bind conserved and highly-homologous phosphorylation sites in other species (*e.g.* mouse, rat, monkey, yeast), in addition to binding the human IRS-1/2 (pSer1101/pSer1149) sites, are within the scope of the present invention. For example, IRS-1/2 antibodies provided also bind the highly homologous Ser1095 and/or Ser1138 sites in mouse IRS-1 and IRS-2, respectively, as well as the homologous Ser 1100 site in rat IRS-1 (SwissProt acc. no. P35570). Additional highly-homologous sites conserved in other species, which are in within the scope of the invention, can readily be identified by standard sequence comparisons, such as using BLAST, with the human IRS-1 and IRS-2 sites disclosed herein.

B. Detection & Profiling Methods

The methods disclosed herein may be employed with any biological sample potentially containing, or suspected of containing, phosphorylated IRS-1 and/or phosphorylated IRS-2. Biological samples taken from human subjects for use in the methods disclosed herein are generally biological fluids such as serum, blood plasma, fine needle aspirant, ductal lavage, bone marrow sample or ascites fluid. In the alternative, the sample taken from the subject can be a tissue sample

(*e.g.*, a biopsy tissue), such as bone marrow or tumor tissue, or a cell lysate, whether or not purified.

In one embodiment, the invention provides a method for detecting phosphorylated IRS-1/2 in a biological sample by (a) contacting a biological sample potentially (or suspected of) containing phosphorylated IRS-1 and/or phosphorylated IRS-2 with at least one detectable reagent that binds to IRS-1 and/or IRS-2 when phosphorylated at Ser1101 and/or Ser1149, respectively, under conditions suitable for formation of a reagent-IRS complex, and (b) detecting the presence of the complex in the sample, wherein the presence of the complex indicates the presence of phosphorylated IRS-1 (Ser1101) and/or phosphorylated IRS-2 (Ser1149) in the sample.

In a preferred embodiment, the reagent is an IRS-1/2 antibody of the invention. In other preferred embodiments, the biological sample has been contacted with at least one PKC inhibitor or a specific PKC theta inhibitor, or is obtained from a subject treated with such inhibitor. As disclosed herein (and discussed below) it has now been discovered that PKC theta phosphorylates IRS-1 at the serine1101 site. Accordingly, changes in IRS-1/2(Ser1101/Ser1149) phosphorylation resulting from contacting a biological sample with a test compound, such as a PKC inhibitor, may be examined to determine effect of such compound. The compound may be a "general" PKC inhibitor that inhibits more than one isoform of PKC (for example, bis-indoleimide), or may be a specific inhibitor of PKC theta. Exemplary inhibitors of PKC include, but are not limited to, Calphostin C, Chelerythrine chloride, Gö 6976, Ro-32-0432, Ro-31-7549, Ro-31-8220, Ro-31-8425, Ro-32-0432, and Rottlerin (commercially available from Calbiochem). Inhibitory compounds may be targeted inhibitors that modulate post-translational activity of PKC, or may be upstream expression inhibitors, such as siRNA or anti-sense inhibitors. In another preferred embodiment, the compound is being tested for

inhibition of PKC activity or expression. Such compound may, for example, directly inhibit PKC activity, or may indirectly inhibit its activity by, e.g., inhibiting another kinase that phosphorylates and thus activates PKC.

Biological samples may be obtained from subjects at risk of, potentially, or suspected of, having a disease or condition involving altered IRS-1/2 expression or activity (*e.g.*, Type 2 diabetes or obesity). For example, samples may be analyzed to monitor subjects who have been previously diagnosed as having diabetes, to screen subjects who have not been previously diagnosed as having diabetes, or to monitor the desirability or efficacy of therapeutics targeted at PKC isoforms, particularly PKC theta. In the case of type 2 diabetes, for example, the subjects will most frequently be adult males and females.

In another embodiment, the invention provides a method for profiling IRS-1 phosphorylation in a test tissue potentially having (or suspected of involving) altered IRS-1/2 activity, by (a) contacting the test tissue with at least one detectable reagent that binds to IRS-1 and/or IRS-2 when phosphorylated at Ser1101 and/or Ser1149, respectively, under conditions suitable for formation of a reagent-IRS complex, (b) detecting the presence of the complex in the test tissue, wherein the presence of the complex indicates the presence of phosphorylated IRS-1 (Ser1101) and/or phosphorylated IRS-2 (Ser1149) in the test tissue, and (c) comparing the presence of phosphorylated IRS-1 and/or IRS-2 detected in step(b) with the presence of phosphorylated IRS-1/2 (Ser1101/Ser1149) in a control tissue, wherein a difference in IRS-1/2 (Ser1101/1149) phosphorylation profiles between the test and control tissues indicates altered IRS-1 and/or IRS-2 activation in the test tissue. In a preferred embodiment, the reagent is a IRS-1/2 antibody of the invention. In other preferred embodiments, the test tissue is diabetic

tissue potentially having (or suspected of involving) altered IRS-1/2 (Ser1101/1149) phosphorylation.

The methods described above are applicable to examining tissues or samples from any disease or condition involving or characterized by altered IRS-1 and/or IRS-2 activity, particularly type 2 diabetes, in which phosphorylation of IRS-1 and/or IRS-2 at Ser1101/1149, respectively, (and possibly other serine residues) has predictive value as to the outcome of the disease or the response of the disease to therapy. It is anticipated that the IRS-1/2 antibodies will have diagnostic utility in a disease characterized by, or involving, altered insulin signaling, or IR or IRS-1/2 activity, or altered IRS-1/2 Ser1101/1149 phosphorylation. The methods are applicable, for example, where samples are taken from a subject has not been previously diagnosed as having type 2 diabetes, nor has yet undergone treatment for diabetes, and the method is employed to help diagnose the disease, or monitor the possible progression of the condition, or assess risk of the subject developing disease involving IRS-1/2 (Ser1101/1149) phosphorylation.

Such diagnostic assay may be carried out prior to preliminary blood evaluation or surgical surveillance procedures. Such a diagnostic assay may be employed to identify patients with activated or inhibited IRS-1 and/or IRS-2 who would be most likely to respond to diabetic therapeutics targeted at activating or inhibiting IRS-1/2 activity. Such a selection of patients would be useful in the clinical evaluation of efficacy of future IRS-1 and/or IRS-2 –targeted therapeutics as well as in the future prescription of such drugs to patients. Alternatively, the methods are applicable where a subject has been previously diagnosed as having a disease involving altered insulin signaling, such as type 2 diabetes or obesity, and possibly has already undergone treatment for the disease, and the method is employed to monitor the progression of the disease

involving IRS-1/2 (Ser1101/1149) phosphorylation, or the treatment thereof.

In another embodiment, the invention provides a method for identifying a compound which modulates phosphorylation of IRS-1/2 in a test tissue, by (a) contacting the test tissue with the compound, (b) detecting the level of phosphorylated IRS-1 and/or IRS-2 in said the test tissue of step (a) using at least one detectable reagent that binds to IRS-1/2 when phosphorylated at Ser1101/Ser1149 under conditions suitable for formation of a reagent-IRS complex, and (c) comparing the level of phosphorylated IRS-1 and/or IRS-2 detected in step(b) with the presence of phosphorylated IRS-1/2 (Ser1101/1149) in a control tissue not contacted with the compound, wherein a difference in IRS-1/2 (Ser1101/1149) phosphorylation levels between the test and control tissues identifies the compound as a modulator of IRS-1/2 phosphorylation. In a preferred embodiment, the reagent is an IRS-1/2 antibody of the invention. In other preferred embodiments, the test tissue is a taken from a subject potentially (or suspected of) having type 2 diabetes and the compound is an IRS-1/2 activator. The compound may modulate IRS-1/2 activity either positively or negatively, for example by increasing or decreasing phosphorylation or expression of IRS-1/2. Alternatively, IRS-1/2 phosphorylation may be monitored to determine the efficacy of a compound targeted at any kinase that phosphorylates IRS-1 (Ser1101) and/or IRS-2 (Ser1149), for example PKC theta (as disclosed herein).

Conditions suitable for the formation of antibody-antigen complexes or reagent-IRS complexes are well known in the art (*see* part (d) below and references cited therein). It will be understood that more than one IRS-1/2 antibody may be used in the practice of the above-described methods. For example, a phospho-IRS-1/2 (Ser1101/Ser1149) antibody and a phospho-specific antibody to another serine, tyrosine, or

threonine phosphorylation site may be simultaneously employed to detect phosphorylation of both sites in one step.

As presently disclosed, it has now been shown that PKC theta phosphorylates IRS-1 at the novel Ser1101 site identified herein. Due to high sequence homology, it is expected that this enzyme will also phosphorylate IRS-2 at Ser1149 (and/or equivalent sites in other species, such as mouse and rat). PKC theta activity is known to be increased in diabetic patients and insulin resistant animals (*see* Chalfant *et al.*, *supra.*; Itani *et al.*, *supra.*; Qu *et al.*, *supra.*), and phosphorylation of IRS-1 at serine residues has been shown to correlate mostly with desensitization of the insulin stimulus (*see* Zick *et al.*, *supra.*) Therefore, PKC theta phosphorylation of IRS-1 at Ser1101 and/or IRS-2 at Ser1149 may represent an important surrogate marker of insulin resistance, which develops as a consequence of Type 2 diabetes. Accordingly, the invention also provides methods for detecting PKC theta activity by assessing phosphorylation of IRS-1 (Ser1101) and/or IRS-2 (Ser1149).

In one embodiment, the invention provides a method for detecting PKC theta activity in a biological sample by (a) contacting a biological with at least one detectable reagent that binds to IRS-1 and/or IRS-2 when phosphorylated at Ser1101 or Ser1149, respectively, under conditions suitable for formation of a reagent-IRS complex, and (b) detecting the presence of the complex in the sample, wherein the presence of the complex indicates the presence of PKC theta activity in the sample. The method may further include the step of (c) comparing the presence of phosphorylated IRS-1 and/or IRS-2 detected in step (b) with the presence of phosphorylated IRS-1 (Ser1101) in a control tissue having known PKC theta activity, wherein a difference in IRS-1 (Ser1101) phosphorylation profiles between the test and control tissues indicates altered PKC theta activity in the test tissue.

In a preferred embodiment, the reagent is an IRS-1/2 antibody of the invention. Biological samples may be obtained from subjects at risk of, potentially, or suspected of having a disease or condition involving or characterized by altered PKC theta expression or activity (*e.g.*, Type 2 diabetes, as in a preferred embodiment). For example, samples may be analyzed to monitor subjects who have been previously diagnosed as having diabetes, to screen subjects who have not been previously diagnosed as having diabetes, or to monitor the desirability or efficacy of therapeutics targeted at PKC theta. In one preferred embodiment, the biological sample has been contacted with at least one PKC inhibitor or PKC theta inhibitor, or is obtained from a subject treated with such inhibitor. Such inhibitors (discussed above) may be test compounds not yet known to in fact inhibit PKC and are being tested for their ability to inhibit PKC activity or expression. Alternatively, they may be compounds known, or anticipated to, so inhibit PKC.

In another embodiment, the invention provides a method for profiling PKC theta activity in a test tissue, by (a) contacting the test tissue with at least one detectable reagent that binds to IRS-1 and/or IRS-2 when phosphorylated at Ser1101 and/or Ser1149, respectively, under conditions suitable for formation of a reagent-IRS complex, (b) detecting the presence of the complex in the test tissue, wherein the presence of the complex indicates the presence of phosphorylated IRS-1 (Ser1101) and/or IRS-2 (Ser1149) in the test tissue, and (c) comparing the presence of phosphorylated IRS-1 and/or IRS-2 detected in step (b) with the presence of phosphorylated IRS-1 (Ser1101) and/or IRS-2(Ser1149) in a control tissue having known PKC theta activity, wherein a difference in IRS-1/2 (Ser1101/Ser1149) phosphorylation profiles between the test and control tissues indicates altered PKC theta activity in the test tissue. In a preferred embodiment, the reagent is an IRS-1 antibody of the invention.

In other preferred embodiments, the test tissue is diabetic tissue suspected of involving altered PKC theta activity.

The methods described above are applicable to examining tissues or samples from any disease or condition involving or characterized by altered PKC theta activity, particularly type 2 diabetes, in which phosphorylation of IRS-1 at Ser1101 and/or IRS-2 at Ser1149 (and both possibly other serine residues) by PKC theta has predictive value as to the outcome of the disease or the response of the disease to therapy. It is anticipated that the IRS-1 antibodies described herein will have diagnostic utility in a disease characterized by, or involving, altered PKC theta activity, and hence resultant altered IRS-1 Ser1101 and/or IRS-2 Ser1149 phosphorylation.

The methods are applicable, for example, where samples are taken from a subject that has not been previously diagnosed as having type 2 diabetes, nor has yet undergone treatment for diabetes, and the method is employed to help diagnose the disease, or monitor the possible progression of the condition, or assess risk of the subject developing the disease. Such diagnostic assay may be carried out prior to preliminary blood evaluation or surgical surveillance procedures. The diagnostic assay may be employed to identify patients with activated or inhibited PKC theta who would be most likely to respond to therapeutics targeted at activating or inhibiting, respectively, PKC theta activity upon IRS-1/2 (Ser1101/Ser1149). Such a selection of patients would be useful in the clinical evaluation of efficacy of future PKC theta targeted therapeutics as well as in the future prescription of such drugs to patients. Alternatively, the methods are applicable where a subject has been previously diagnosed as having a disease involving altered PKC theta activity, such as type 2 diabetes, and possibly has already undergone treatment for the disease, and the method is employed to monitor the progression of the

disease by monitoring IRS-1 (Ser1101) phosphorylation and/or IRS-2 (Ser1149) phosphorylation, or the treatment thereof.

In another embodiment, the invention provides a method for identifying a compound which modulates PKC theta activity in a test tissue, by (a) contacting the test tissue with the compound, (b) detecting the level of phosphorylated IRS-1/2 in said test tissue of step (a) using at least one detectable reagent that binds to IRS-1 and/or IRS-2 when phosphorylated at Ser1101 and/or Ser1149, respectively, under conditions suitable for formation of a reagent-IRS complex, and (c) comparing the level of phosphorylated IRS-1/2 detected in step(b) with the presence of phosphorylated IRS-1 (Ser1101) and/or IRS-2 (Ser1149) in a control tissue not contacted with the compound having known PKC theta activity, wherein a difference in IRS-1/2 (Ser1101/Ser1149) phosphorylation levels between the test and control tissues identifies the compound as a modulator of PKC theta activity. In a preferred embodiment, the reagent is an IRS-1 antibody of the invention. In other preferred embodiments, the test tissue is a taken from a subject suspected of having type 2 diabetes and the compound is a PKC theta inhibitor.

The compound may modulate PKC theta activity either positively or negatively, for example by increasing or decreasing phosphorylation or expression of IRS-1 and/or IRS-2. IRS-1 (Ser1101) phosphorylation and activity may be monitored, for example, to determine the efficacy of an anti-PKC theta therapeutic, *e.g.* a PKC theta inhibitor. Alternatively, IRS-1 (Ser1101) and/or IRS-2 (Ser1149) phosphorylation may be monitored to determine the efficacy of a compound targeted at any kinase that activates or inhibits PKC theta.

C. Immunoassay Formats & Diagnostic Kits

Assays carried out in accordance with methods of the present invention may be homogeneous assays or heterogeneous assays. In a homogeneous assay the immunological reaction usually involves a IRS-1/2-specific reagent (*e.g.* a IRS-1/2 antibody of the invention), a labeled analyte, and the sample of interest. The signal arising from the label is modified, directly or indirectly, upon the binding of the antibody to the labeled analyte. Both the immunological reaction and detection of the extent thereof are carried out in a homogeneous solution. Immunochemical labels that may be employed include free radicals, radioisotopes, fluorescent dyes, enzymes, bacteriophages, coenzymes, and so forth.

In a heterogeneous assay approach, the reagents are usually the specimen, an IRS-1/2-specific reagent (*e.g.*, the IRS-1/2 antibody of the invention), and suitable means for producing a detectable signal. Similar specimens as described above may be used. The antibody is generally immobilized on a support, such as a bead, plate or slide, and contacted with the specimen suspected of containing the antigen in a liquid phase. The support is then separated from the liquid phase and either the support phase or the liquid phase is examined for a detectable signal employing means for producing such signal. The signal is related to the presence of the analyte in the specimen. Means for producing a detectable signal include the use of radioactive labels, fluorescent labels, enzyme labels, and so forth. For example, if the antigen to be detected contains a second binding site, an antibody which binds to that site can be conjugated to a detectable group and added to the liquid phase reaction solution before the separation step. The presence of the detectable group on the solid support indicates the presence of the antigen in the test sample. Examples of suitable immunoassays are the

radioimmunoassay, immunofluorescence methods, enzyme-linked immunoassays, and the like.

Immunoassay formats and variations thereof, which may be useful for carrying out the methods disclosed herein, are well known in the art. *See generally* E. Maggio, Enzyme-Immunoassay, (1980) (CRC Press, Inc., Boca Raton, Fla.); *see also, e.g.,* U.S. Pat. No. 4,727,022 (Skold *et al.,* "Methods for Modulating Ligand-Receptor Interactions and their Application"); U.S. Pat. No. 4,659,678 (Forrest *et al.,* "Immunoassay of Antigens"); U.S. Pat. No. 4,376,110 (David *et al.*, "Immunometric Assays Using Monoclonal Antibodies"). Conditions suitable for the formation of reagent-antibody complexes are well described. *See id.* Monoclonal antibodies of the invention may be used in a "two-site" or "sandwich" assay, with a single cell line serving as a source for both the labeled monoclonal antibody and the bound monoclonal antibody. Such assays are described in U.S. Pat. No. 4,376,110. The concentration of detectable reagent should be sufficient such that the binding of phosphorylated IRS-1 is detectable compared to background.

IRS-1/2 antibodies disclosed herein may be conjugated to a solid support suitable for a diagnostic assay (*e.g.*, beads, plates, slides or wells formed from materials such as latex or polystyrene) in accordance with known techniques, such as precipitation. Antibodies of the invention, or other IRS-1 binding reagents, may likewise be conjugated to detectable groups such as radiolabels (*e.g.*, ^{35}S, ^{125}I, ^{131}I), enzyme labels (*e.g.*, horseradish peroxidase, alkaline phosphatase), and fluorescent labels (*e.g.*, fluorescein) in accordance with known techniques.

IRS-1/2 antibodies of the invention may also be used in a flow cytometry assay to determine the activation status of IRS-1/2 in patients before, during, and after treatment with a drug targeted at inhibiting IRS-1/2 phosphorylation at Ser1101/1149. Inhibitors of PKC theta are one example of such phosphorylation inhibitors. For example, bone marrow

cells or peripheral blood cells from patients may be analyzed by flow cytometry for IRS-1/2 phosphorylation, as well as for markers identifying various hematopoietic cell types. In this manner, IRS-1/2 activation status of the diseased cells may be specifically characterized. Flow cytometry may be carried out according to standard methods. *See*, *e.g.* Chow *et al.*, *Cytometry (Communications in Clinical Cytometry) 46:* 72–78 (2001). Briefly and by way of example, the following protocol for cytometric analysis may be employed: fixation of the cells with 2% paraformaldehyde for 20 minutes at 37 °C followed by permeabilization in 90% methanol for 30 minutes on ice. Cells may then be stained with the primary IRS-1 antibody, washed and labeled with a fluorescent-labeled secondary antibody. The cells would then be analyzed on a flow cytometer (*e.g.* a Beckman Coulter EPICS-XL) according to the specific protocols of the instrument used. Such an analysis would identify the presence of phosphorylated IRS-1 and/or IRS-2 in a cell of interest and reveal the drug response on the targeted IRS-1 protein or kinase (*e.g.* PKC theta).

Diagnostic kits for carrying out the methods disclosed above are also provided by the invention. Such kits comprise at least one detectable reagent that binds to IRS-1 and/or IRS-2 when phosphorylated at Ser1101 and/or Ser1149, respectively. In a preferred embodiment, the reagent is an IRS-1/2 antibody of the invention. In one embodiment, the invention provides a kit for the detection of phosphorylated IRS-1 (Ser1101) and/or IRS-2 (Ser1149) in a biological sample comprising (a) at least one IRS-1/2 antibody of the invention (*i.e.* a phospho-specific antibody that binds phospho-IRS-1/2 (Ser1101/Ser1149)) and (b) at least one secondary antibody conjugated to a detectable group. The reagents may also include ancillary agents such as buffering agents and protein stabilizing agents, *e.g.*, polysaccharides and the like. The diagnostic kit may further include, where necessary, other members of the signal-

producing system of which system the detectable group is a member (*e.g.*, enzyme substrates), agents for reducing background interference in a test, control reagents, apparatus for conducting a test, and the like.

In another embodiment, the invention provides a kit for the detection of PKC theta activity in a biological sample comprising (a) at least one IRS-1/2 antibody of the invention (*i.e.* a phospho-specific antibody that binds phospho-IRS-1 (Ser1101) and/or phospho-IRS-2 (Ser1149) and (b) at least one secondary antibody conjugated to a detectable group. Ancillary agents as described above may likewise be included. The test kit may be packaged in any suitable manner, typically with all elements in a single container along with a sheet of printed instructions for carrying out the test.

The following Examples are provided only to further illustrate the invention, and are not intended to limit its scope, except as provided in the claims appended hereto. The present invention encompasses modifications and variations of the methods taught herein which would be obvious to one of ordinary skill in the art.

EXAMPLE 1

Production of an IRS-1/2 (Ser1101/Ser1149) Phospho-specific Antibody

A previously unknown IRS-1 phosphorylation site, serine 1101, was identified as described above by predictive analysis of the human IRS-1 protein sequence using the ScanSite program. Yaffe *et al.*, *supra.* A 13 amino acid phospho-peptide antigen, CRRRHS***S**ETFSST (SEQ ID NO: 11) (where ***S** = phosphoserine), corresponding to residues 1095-1107 of human IRS-1 (*see* SEQ ID NO: 1), was constructed according to standard synthesis techniques using a Rainin/Protein Technologies, Inc., Symphony peptide synthesizer. *See* ANTIBODIES: A LABORATORY MANUAL,

supra.; Merrifield, *supra.* This peptide antigen also corresponds to highly homologous residues 1143-1155 of human IRS-2 (see SEQ ID NO: 2) (as well as the highly homologous sites in murine IRS-1 and IRS-2; *see* Figs. 13-14).

This peptide was coupled to KLH, and rabbits were injected intradermally (ID) on back with antigen in complete Freunds adjuvant (500 μg antigen per rabbit). The rabbits were boosted with same antigen in incomplete Freund adjuvant (250 μg antigen per rabbit) every three weeks. After the fifth boost, the bleeds were collected. The sera were purified by Protein A-sepharose affinity chromatography as previously described (*see* ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor, *supra.*). Further purification steps were performed using adsorption of non-specific material to nonphosphopeptide affinity column, followed by elution of reactive material from a phosphopeptide affinity column at low pH, as follows. The eluted immunoglobulins were loaded onto CRRRHSSETFSST-resin Knotes column. The flow through fraction was collected and applied onto CRRRHS*****SETFSST –resin column. After washing the column extensively, the phospho-IRS-1/2 (Ser1101/Ser1149) antibodies were eluted, dialyzed, and kept in antibody storage buffer.

Antibodies were characterized by ELISA against phospho- and nonphosphopeptides to determine the extent of phosphospecificity and by Western blotting to examine specificity against whole cell extracts, as described in detail below.

Characterization of p-IRS-1/2 (Ser1101/Ser1149) Antibodies Against Phosphorylated Ser1101 and Ser1149 , in Human IRS-1/2.

To characterize the polyclonal antibodies raised against the phosphorylated peptide described above, Western blots with three cell types were used: NIH3T3 L1 differentiated adipocytes, L6 differentiated myocytes and CHO cells overexpressing the insulin receptor and IRS-1.

The different cell types were stimulated with insulin, TPA, PDGF and TNF α, all factors known to induce serine phosphorylation of IRS-1 through the activation of different pathways.

Figure 3 shows the time course of phosphorylation of IRS-1 at Ser1101, Ser 330, and Ser307 upon stimulation with insulin in CHO cells overexpressing IRS-1 and the insulin receptor. Phosphorylation of a previously described site in IRS1, Ser612, as well as activation of the Akt and MAPK pathways is shown to follow the same induction kinetics as Ser1101, 307 and 330. Since fat and muscle tissues are among the most important insulin responsive tissues in the body, phosphorylation of IRS-1 at Ser1101, 330 and 307 in adipocytes and myocytes was further examined. 3T3L1 adipocytes were treated with insulin for 5 and 30 minutes and L6 myocytes were treated with TNF α and insulin for 15 minutes. IRS-1 was immunoprecipitated and immunoblotted with phospho-IRS1 antibodies against Ser1101, Ser330 (only in muscle cells) and Ser307, as well as total IRS-1 antibodies.

The results shown in Figures 4 and 5 demonstrate that Ser1101 is phosphorylated by insulin treatment (fat and muscle cells) and by TNF α treatment (muscle cells). Upon insulin treatment phosphorylation at Ser307 occurred in both cell types, but at Ser330 only in myocytes, whereas both sites were not affected by TNF α treatment. Analysis of total IRS1 protein and phosphotyrosine-IRS-1 served as controls to indicate equal loading and to verify that the stimuli applied worked as expected.

To determine the specificity of the IRS-1/2 phospho-(Ser1101/Ser1149) antibodies, expression constructs encoding epitope (HA) tagged wild-type IRS-1 protein, or IRS-1 containing an alanine substitution at position 1101 were transfected to NIH 3T3 cells, according to standard methods (*See* Qiagen Polyfect® Transfection Reagent Handbook, Sept. 2000).

These cell were stimulated with insulin and HA-IRS-1 proteins were immunoprecipitated using an anti-HA antibody. The immunoprecipitated material was then immunoblotted using the phospho-IRS-1/2 antibody. Figure 6 shows that the phospho IRS-1/2 (Ser1101/Ser1149) antibody detected the wild-type protein, but failed to detected IRS-1 when Ser1101 was mutated to alanine. This demonstrates that the phospho IRS-1/2 antibody recognizes specifically phosphorylated Ser1101 in IRS-1. Anti-HA antibodies were used to control for the amounts of total IRS-1 protein immunoprecipitated and immunoblotted in this experiments, and phosphotyrosine immunoblot indicated that the insulin treatment worked as expected leading to tyrosine phosphorylation of IRS-1.

We next examined whether IRS-1 phosphorylation at Ser1101 would correlate with the insulin resistance state that typically occurs in obese Zucker rats. Total IRS-1 protein was immunoprecipitated from liver extracts of lean or obese Zucker rats, and immunoblotted with the phospho-IRS-1 Ser1101 antibody. Figure 7 shows an increased level of phosphorylation of IRS-1 at Ser1101 in the liver of obese, but not lean Zucker rats. These results suggests that phosphorylation of IRS-1 at Ser1101 is a marker of obesity –induced insulin resistance.

Because of the high homology (and near identity) between the Ser1101 site in IRS-1 and Ser1149 site in IRS-2, the ability of polyclonal antibodies raised against the phosphorylated peptide to also detect the homologous phosphorylated Ser1149 in IRS-2 was examined, using a CHO cell line overexpressing the IRS-2 protein. Figure 8 shows that insulin stimulated the phosphorylation of IRS-2 at Ser1149 reaching maximal levels after one hour of exposure to insulin. These results indicate that the phospho-IRS-1/2 antibodies also specifically detect IRS-2 when phosphorylated at Ser 1149. The antibodies also bind the highly homologous and equivalent Ser1095 and Ser 1138 sites in mouse

IRS-1 and IRS-2, respectively, and the equivalent IRS-1 (Ser1100) site in rats.

EXAMPLE 2

Phosphorylation of IRS-1 (Ser1101) by Protein Kinase C theta

In order to analyze the potential signaling pathways leading to phosphorylation of Ser1101, CHO-IR/IRS-1 cells were stimulated with insulin, PDGF and with the activator of protein kinase C, TPA, and IRS-1 proteins were immunoprecipitated with a specific IRS-1 antibody followed by immunoblotting with the IRS-1/2 (Ser1101/Ser1449) phospho-antibodies described in Example 1. Figure 9 shows a robust induction of phosphorylation at Ser1101, particularly upon PKC activation (left panel). As expected, only insulin treatment induced tyrosine phosphorylation of the insulin receptor and IRS-1 (right panel), but all treatments induced MAPK and phosphorylation of PKCs. Also, as previously reported, only insulin and PDGF induced the activation of Akt. This results suggest that despite being selected as an Akt site by ScanSite, Ser1101 is not dependent on Akt activation, but most likely on PKC activation. This is supported by the fact that phosphorylation at Ser1101 is inhibited by the PKC inhibitor Bisindoleimide, but not by inhibitors of the PI3K/ Akt pathway, wortmannin and LY294002 (Fig. 10).

Insulin-dependent phosphorylation of IRS-1 at Ser1101 is also not affected by inhibitors of the MEK/MAPK pathway (PD98059), PKA and MSK1 kinases (H89), CaM kinases (KN62), Src tyrosine kinases (Genistein and PP2). Ser1101 is somewhat reduced by SU6686, an inhibitor of the insulin receptor tyrosine kinase (Fig. 10). The results indicate that upon different types of stimuli, insulin, PDGF, TNF-α, TPA, etc., IRS-1 becomes phosphorylated at the previously unknown site Ser1101, as well as Ser307 and Ser330. In the case of Ser1101, the

evidence indicates a PKC isoform being involved as the kinase directly phosphorylating Ser1101 or participating in the pathway leading to Ser1101 phosphorylation. Multiple lines of evidence have suggested that isoform-selective activation of PKC phosphorylates and down-regulates IRS-1, or other components of the insulin pathway. The strong link between insulin resistance and increased lipid availability has led to the proposal that accumulation of lipid metabolites, via activation of protein kinase C leads to dysregulated insulin signaling (*see* Pan *et al.*, *Diabetes 46:* 983-988 (1997)). Recent studies have linked an increased amount and activities of certain PKC isoforms, particularly PKC theta, in skeletal muscle of insulin resistant human patients and of insulin-resistant obese Zucker rats (*see*, Itani *et al.*, *supra.*; Qu *et al.*, *supra.*; Chalfant *et al.*, *supra.*)

However, despite the fact that Ser1101 was predicted by ScanSite as an Akt substrate, the results shown in Figures 9 & 10 suggest that Akt cannot phosphorylate this site *in vivo*. Accordingly, the phosphorylation of IRS-1 (Ser1101) by PKC theta and Akt *in vitro* was examined. Kinase reactions were performed with active Akt and multiple PKC isoform kinases using a GST-IRS-1 C-terminal fragment containing IRS-1 and full-length IRS-1 pulled down from unstimulated CHO-IR/IRS-1 cells. As shown in Figure 11, PKC theta is the only PKC isoform that robustly phosphorylated the GST –IRS-1 fusion protein substrate at Ser1101. The Akt kinase could not effectively phosphorylate IRS-1 at Ser1101, despite the occurrence of this residue within a putative Akt motif. This result demonstrates that Ser1101 is a substrate of PKC theta *in vitro*.

To verify whether PKC theta controls phosphorylation of IRS-1 Ser1101 *in vivo*, HA-tagged IRS-1 wild-type expression constructs were co-transfected (according to standard methods, see Qiagen Polyfect® Handbook, *supra.*) with constructs expressing a constitutively activated version of PKC theta and a dominant negative PKC theta mutant. Figure

12 shows that constitutively activate PKC theta induced the phosphorylation of IRS-1 at Ser1101 without the need of insulin stimulation. PKC theta induced phosphorylation of IRS-1 Ser1101 also correlated with a reduction in tyrosine phosphorylation of IRS-1, which is indicative of IRS-1 inactivation. Consistently, dominant negative PKC theta blocked insulin-induced phosphorylation of IRS-1 at Ser1101. Altogether the combined results shown in Figures 8-11 demonstrate that the IRS-1 Ser1101 site is substrate of PKC theta in vitro and *in vivo*. Given the near-identity of the corresponding IRS-1 Ser1149, it is expected that this site will also be a substrate of PKC theta (as well as equivalent sites in other species such as rat, mice). These results indicate that Ser1101 (and Ser1149) represents a novel marker of PKC theta activity in samples from insulin resistant patients and a marker to monitor the activity of PKC theta specific inhibitors, which could potentially be used to treat insulin resistance.

EXAMPLE 3

Production of an IRS-1/2 (Ser1101/Ser1149) Phospho-specific Monoclonal Antibody

Phospho-IRS-1/2(Ser1101/Ser1149)–specific monoclonal antibodies may be produced from spleen cells of the immunized BALB/c mouse described in Example 1, above, following standard procedures (Harlow and Lane, 1988). Briefly, the mouse spleen is fused to SP2/0 mouse myeloma fusion partner cells according to the protocol of Kohler and Milstein (1975). Colonies originating from the fusion are screened by ELISA for reactivity to the phospho-peptide and non-phospho-peptide and by Western blot analysis. Colonies found to be positive by ELISA to the phospho-peptide while negative to the non-phospho-peptide may be further characterized by Western blot analysis. Colonies found to be positive by Western blot analysis are then subcloned by limited dilution.

Mouse ascites are produced from positive clones obtained from subcloning. Clones are selected for phospho-specificity by ELISA and by Western blot analysis using cell culture supernatant. Selected positive clones are then subcloned to produce final desired clones producing phospho-IRS-1/2 (Ser1101/Ser1149)-specific monoclonal antibodies.

Ascites fluid from clones obtained from the IRS-1/2 fusion may be further tested by Western blot analysis. The ascites fluid will likely give similar results on Western blot analysis as observed with the cell culture supernatant, indicating phospho-specificity on IRS-1/2 -induced 3T3L1 adipocytes and/or L6 differentiated myocyte cells, for example.